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Evidence for a *Pneumocystis carinii* Flo8-like Transcription Factor: Insights into Organism Adhesion

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Abstract

Pneumocystis carinii (*Pc*) adhesion to alveolar epithelial cells is well established and is thought to be a prerequisite for initiation of *Pneumocystis* pneumonia. *Pc* binding events occur in part through the major *Pc* surface glycoprotein Msg, as well as an integrin-like molecule termed PcInt1. Recent data from the *Pc* sequencing project also demonstrate DNA sequences homologous to other genes important in *Candida* spp. binding to mammalian host cells, as well as organism binding to polystyrene surfaces and in biofilm formation. One of these genes, *flo8*, a transcription factor needed for downstream cAMP/PKA-pathway-mediated activation of the major adhesin/flocculin Flo11 in yeast, was cloned from a *Pc* cDNA library utilizing a partial sequence available in the *Pc* genome database. A CHEF blot of *Pc* genomic DNA yielded a single band providing evidence this gene is present in the organism. BLASTP analysis of the predicted protein demonstrated 41% homology to the *Saccharomyces cerevisiae* Flo8. Northern blotting demonstrated greatest expression at pH 6.0–8.0, pH comparable to reported fungal biofilm milieu. Western blot and immunoprecipitation assays of PcFlo8 protein in isolated cyst and trophozoite life forms confirmed the presence of the cognate protein in these *Pc* life forms. Heterologous expression of *Pcflo8* cDNA in *flo8*[−] (deficient) yeast strains demonstrated the *Pcflo8* was able to restore yeast binding to polystyrene and invasive growth of yeast *flo8*[−] cells. Furthermore, *Pcflo8* promoted yeast binding to HEK293 human epithelial cells, strengthening its functional classification as a Flo8 transcription factor. Taken together these data suggests that PcFlo8 is expressed by *Pc* and may exert activity in organism adhesion and biofilm formation.

Keywords

Pneumocystis; Flo8; cell adhesion; pathogenesis

INTRODUCTION

Pneumocystis jirovecii inflicts severe and life-threatening pneumonia in patients with impaired immune systems, particularly in individuals with HIV infection [1]. Although

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CONFLICT OF INTEREST STATEMENT

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intensive efforts have been undertaken to culture *Pc in vitro*, these attempts have yielded only modest and unreliable levels of replication [2]. This inability to propagate and genetically manipulate *Pc* has hindered basic cell biology investigations of this important pathogen.

Due to these challenges, little has been learned of *Pc* adhesion to host lung epithelium as well as the potential formation of biofilm. Our recent work has shown that *Pc* binding to extracellular matrix components and lung epithelial cells induces expression of a PcSte20 kinase implicated in pseudohyphal growth and mating, as well as the PcAce2 transcription factor which exerts effects on cell wall remodeling events prior to cell replication [3,4]. Others have shown that *Pneumocystis jirovecii* may form potential biofilm structures in close approximation to host lung epithelium and that *Pc* biofilm formation plays an important role in *Pc* resistance to antifungal therapeutic agents [5,6].

To better understand the possible role of adhesion events in *Pc* biology, we sought to characterize a possible homologue of the yeast Flo8 transcription factor. The Flo8 transcription factor in yeast has been shown to bind the Flo11 promoter causing transcription of this major yeast cell surface glycoprotein involved in organism adherence. In yeast, the presence of Flo11 protein has also been linked to the formation of biofilm formation and adhesion to polystyrene surfaces [7]. *C. albicans* Flo8 is also required for hyphal development, binding to epithelial cells, and for virulence in animal models [8,9]. Upon alignment of *S. cerevisiae* FLO8 nucleotide sequence to *Pc* Expressed Sequence Tags (ESTs) from the *Pc* sequencing project and performing BLASTP analysis, an open reading frame with homology to the amino-terminal end of *S. cerevisiae* Flo8 was identified. Accordingly we performed an in-depth characterization of the *Pc* Flo8 transcription factor involving DNA, RNA, protein, and yeast heterologous expression tools to gain new insight into this novel molecule expressed by *Pneumocystis*.

MATERIALS AND METHODS

Reagents and strains

Pc organisms were originally derived from American Type Culture Collection (ATCC, Manassas, VA) and stocks were propagated and purified from corticosteroid-treated rats as reported previously [10]. Unless otherwise noted, all reagents were obtained from Sigma-Aldrich (St. Louis, MO). Standard yeast genetics and molecular biology techniques were implemented to generate the yeast strains and plasmids described in this report.

Pc nucleic acid isolation

Total RNA for cDNA generation described below, was isolated from *Pc* using TRIzol® reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

Identification of the complete Pcflo8 cDNA

The *Pneumocystis carinii* Genome Project Database (<http://pgp.cchmc.org>) was searched with the keyword "flo8". Upon this search, partial DNA sequences with homology to *S. cerevisiae* FLO8 sequences were identified. Of these, we focused on a potential 934-

nucleotide partial sequence do to its highest homology with the yeast Flo8 transcription factor. Rapid amplification of cDNA ends (RACE) procedure was employed accordingly (GeneRacer® Kit, Life Technologies) to obtain the full-length 2184-bp cDNA sequence. This gene is listed in the National Center for Biotechnological Information (NCBI) under the accession number KJ790261. MacVector™ software (Accelrys Software, Cary, NC) was employed for protein alignments and sequence analysis.

Pcflo8 chromosomal hybridization

To verify that the full-length *Pcflo8* coding sequence was present in the *Pc* genome, a 682-bp probe was amplified from the full-length DNA template and hybridized to *Pc* chromosomes separated by contour-clamped homogenous field (CHEF) electrophoresis blot as described previously [11]. The *Pcflo8* probe was labeled using [α -³²P] dATP by random primer method (Rediprime System, GE Healthcare Life Sciences, Pittsburg, PA). The CHEF membrane was incubated with the probe (1.5×10^6 cpm/ml) at 60°C for 1 h, washed three times at room temperature for 40 min in 2X SSC buffer containing 0.05% SDS, washed twice at 50°C for 40 min in 2X SSC buffer containing 0.1% SDS, and examined by autoradiography.

Assessment of *Pc* Pcflo8 transcription

The expression of *Pcflo8* under pH conditions mimicking external as well as host lung physiological pH were analyzed by Northern blotting. Total *Pc* life forms were maintained over 2 h at pH levels 4.0–8.0 in 1.0 ml of Ham's F-12 tissue culture medium supplemented with 10% fetal bovine serum. After 2 hours, total RNA was isolated as described. Equal RNA (10.0 µg) was separated through a 1.0% agarose gel in the presence of 2.2 M formaldehyde, transferred to nitrocellulose, and probed with the 682-bp radiolabeled *Pcflo8* amplicon. Following Northern blotting hybridization (Clontech, Mountain View, CA), membranes were washed four times and visualized by autoradiography.

Antibody generation to the predicted PcFlo8 transcription factor and immunoblotting for PcFlo8 protein

To evaluate protein levels of the PcFlo8 transcription factor in isolated *Pc* cystic and trophic forms; a polyclonal antibody was generated to a 19-residue peptide (NSPNSPTDGPLSQNN TSR) corresponding to amino acids 509–527 of the mature PcFlo8 protein. This region was chosen for its high antigenicity index as determined by MacVector™ software (Accelrys Software). Polyclonal antiserum was generated in rabbits (Bethyl Laboratories Inc., Montgomery, TX). Purification of reactive IgG antibody and coupling to horseradish peroxidase (HRP), along with confirmation of antibody specificity was performed by Bethyl Labs. To assess the abundance of PcFlo8 in the isolated life forms of *Pc*, freshly isolated *Pc* organisms were separated into cystic and trophic forms by differential filtration through 3-µm filters, which allow passage of trophic forms, but retain cysts. This differential procedure yielded 99.5% pure trophic populations and preparations that were enriched in *Pc* cysts by 40-fold [12]. The separated populations were lysed in radio-immunoprecipitation assay (RIPA) buffer with protease inhibitors added (cOmplete, EDTA-free cocktail tablets, Roche USA, Branford, CT). Determination of PcFlo8

transcription levels in the separated life forms of *Pc* was achieved in two ways. First, the respective total *Pc* life form protein in RIPA buffer (20 µg) was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. PcFlo8 protein presence was then measured by immunoblotting with the PcFlo8-HRP IgG antibody (0.10 µg/ml) for 2 h. In parallel, total *Pc* protein lysates (200 µg) were immunoprecipitated with anti-PcFlo8-IgG (0.40 µg/ml) for 2 h at 4°C, end-over-end (EOE). Next, Protein A-Sepharose (50% slurry) was added and tubes incubated 1 h at 4°C with mixing. After five washes with 1X phosphate buffered saline (PBS), Laemmli sample buffer was added to the tubes and the eluents separated by PAGE gels as above. The resolved gels were stained with Bio-Safe™ Coomassie Stain (Bio-Rad, Hercules, CA) to detect the precipitated PcFlo8 antibody-PcFlo8 protein complexes. For both of these studies, to assure that the anti-PcFlo8 antibody was not reacting with any host cell contaminants, identical concentrations of healthy rat lung proteins were assayed in a similar fashion.

PcFlo8 immune localization in *Pneumocystis*

To next evaluate the presence of the PcFlo8 transcription factor in *Pc* life forms, an immunofluorescence microscopy protocol for yeast cells was utilized (http://www.colorado.edu/mcldb/odorizzilab/Odorizzi_Lab/Welcome_files/Immunofluorescence%20of%20Yeast.pdf) followed by a DAPI nuclear staining method (http://www.hopkinsmedicine.org/institute_basic_biomedical_sciences/research_centers/high_throughput_biology_hit/technology_center_networks_pathways/pdfs/protocols/MELUH_Quick_DAPI_Staining_of_Yeast.pdf). To detect the localization of the transcription factor in *Pc*, the same antibody used in the immunoprecipitation assay described above was employed. First, purified *Pc* life forms were fixed in 3.7% formaldehyde at RT for 2 h. Life forms were then washed 2X with PBS. Next, to permeabilize the trophic cell wall, EtOH was added at a final concentration of 70% and allowed to incubate for 40 min at RT. Subsequently, after washing and re-suspending the life forms in PBS, trophic forms were applied to poly-L-lysine coated slides. The slides were incubated with the primary PcFlo8 IgG affinity-purified antibody (1:1000 dilution of 1.0 mg/ml stock) for 2 h at room temperature. The slides were then washed three times with 3% bovine serum albumin (BSA) in PBS over 5 min. An anti-rabbit IgG antibody produced in goat conjugated to Alexa Fluor® 488 (Life Technologies), (1:100 dilution) was added to the slides and incubated for an additional hour. To determine if the PcFlo8 transcription factor might co-localize in the nucleus, slides were washed 3X with PBS, and Prolong Diamond Antifade Mountant (Life Technologies) containing 75 ng/ml 4',6-diamidino-2-phenylindole (DAPI) was added. After applying a coverslip, slides were visualized under oil immersion using an Olympus IX70 microscope (100X magnification) with phase contrast and the respective filters for determining PcFlo8 localization.

Gene transcription analysis by PcFlo8

Yeasts cells were grown overnight in 2% galactose synthetic media dropout media (-URA), and total RNA was isolated with TRIzol® as noted above. All RNA samples were treated with RNase free DNase (Life Technologies). RT-PCR experiments, utilizing equal amounts of total RNA (200 ng) was used to generate first-strand cDNA synthesis with SuperScript® III Reverse Transcriptase (Life Technologies) according to the manufacturer's

recommendations. After first-strand synthesis, cDNA was used for semiquantitative PCR. Oligonucleotide primers used were as follows: ACT1; 5'-TGAACACGGTATTGTCACC-3', and 5'-AACGGCTTGGATGGAAACG-3', FLO11; 5'-ACTTTGGATGTGACTTCCG-3', and 5'-GCTGTGAAATCAGTTGGGTTG-3'.

Polystyrene adherence

Adherence of *flo8* yeast with the control vector (*flo8* + pYES2.1/V5-His/*lacZ*), wild-type cells with the same control plasmid (*FLO8* + pYES2.1/V5-His/*lacZ*) or *flo8* yeast cells with *Pcflo8* cDNA (*flo8* + pYES2.1/*Pcflo8*) to polystyrene surfaces was performed as described previously [7]. Yeast were grown overnight at 30°C in synthetic complete (SC) media lacking uracil to maintain selection of plasmids and with 2% galactose to induce the *GALI* promoter and downstream gene transcription [4]. After yeast cells reached an OD₆₀₀ nm between 0.5 and 1.5, the organisms were collected, washed in growth media, and re-suspended to OD₆₀₀ nm of 1.0 in yeast growth media. Cell suspensions (100 µl) were transferred in duplicate to 96 well polystyrene microtiter plates (Falcon Microtest flat bottom plate, 35-1172, Becton, Dickinson and Co., East Rutherford, NJ). Cells were then incubated for 0–180 min, stained with crystal violet, and the wells washed repeatedly with water.

Agar invasion

To determine whether *Pcflo8* could restore the wild-type invasive agar phenotype, we used a method as previously reported [14]. All strains tested were grown overnight at 30°C in SC media with 2% galactose and without uracil. The following day, equal numbers of yeast cells were plated on SC agar plates and incubated for 2 days at 30°C. Any non-adherent cells were washed with running water. Photographs were taken prior to and before plate washing.

Binding of yeast to human epithelial cells

Studies to determine if *Pcflo8* expression in *flo8* yeast cells could support organism adherence to human epithelial cells were conducted as described previously [8]. After yeast cells were inoculated onto HEK293 epithelial cell monolayers, the plates were spun at 500 x g for 5 min to synchronize infection. Plates were then incubated for 1 h at 37°C. Initial inoculation number was confirmed by colony counting on SC media. 1X PBS was used to wash away non-adherent yeast cells. Next trypsin was added to the wells and the yeast cells re-suspended in 1X PBS. This cell suspension was then applied to SC media containing 2% galactose without uracil and colony forming units determined after culture.

Statistical analysis

All the data shown are expressed as the mean ± SEM derived from multiple experimental runs. Statistical differences between various data groups were first determined using analysis of variance (ANOVA) and subsequently Student's T-tests as indicated. The corresponding non-parametric statistics were applied when data were not distributed in a Gaussian manner. Statistical testing was performed using GraphPad Prism version 5.0b software, and statistical differences were considered to be significant if $P < 0.05$.

RESULTS

The predicted *PcFlo8* shares significant homology with *S. cerevisiae FLO8*. Using a combination of *in silico* search of the *Pc* genome database and RACE PCR techniques to extend the partial sequences identified, we were able to successfully isolate a full-length coding sequence of the *Pc PcFlo8* cDNA encompassing 2184-bp. The predicted *PcFlo8* protein has an estimated size of ~82.7 kDa and pI of 10.07. Protein alignment of *PcFlo8* with *S. cerevisiae Flo8* reveals approximately 41% homology by BLASTP analysis (Figure 1, A.). Interestingly, at amino acid positions 242–245 of *PcFlo8*, a cAMP-dependent protein kinase phosphorylation site was observed. In *S. cerevisiae*, binding of *Flo8* to the *Flo11* promoter is regulated by *Tpk2*, one of three subunits of protein kinase A (PKA). *Flo11* represents the major flocculin in yeast, which is important in adherence, flocculation, and invasive growth. Regulation of *Flo8* by *Tpk2* occurs through phosphorylation of this c-AMP protein kinase site in yeast [15]. It is noteworthy that this important regulatory element appears to be well conserved in members of the *Pneumocystis* genus. Indeed, when the yeast *Tpk2* protein is used in BLASTP analysis against both the *P. jirovecii* and *P. murina* genome databases, (links: <http://genome.jgi.doe.gov/Pneji1/Pneji1.home.html> and http://www.broadinstitute.org/annotation/genome/Pneumocystis_group.2/MultiHome.html), a *Tpk2*-like protein with high homology (77%, and 78%, respectively) were found in both. *PcFlo8*, similar to yeast *Flo8*, also contains a LUFS domain (LUG/LUH, *Flo8*, single-stranded DNA binding protein) (Figure 1, B.) [16]. LUG serves as a master transcriptional regulator during plant development and morphogenesis and more recently, studies of *C. albicans Flo8* indicate that this gene exerts activity in hyphal growth [9]. Also within the LUFS domain, *PcFlo8* appears to exhibit high homology with the yeast *Flo8 LisH* (Lissencephaly type-1) domain (Figure 1, B.) [17]. These motifs are highly thermodynamically stable dimerization domains that when coupled with other *LisH*-containing transcription factors, can tightly regulate downstream gene regulation [18]. Taken together, these data suggest that the *PcFlo8* protein is a homolog of the *Flo8*-fungal and yeast transcription factor family. Furthermore, with the discovery of this transcription factor in *Pc*, along with a *Tpk2* yeast-like subunit homolog, the potential exists that this gene may also regulate adhesion in *Pneumocystis*.

The *Pcflo8* sequence is present in the *Pc* genome

Since the *Pcflo8* was derived by PCR from *Pc* organisms purified from rat lung, it was essential to verify that the derived sequences were present in the *Pc* genome. To accomplish this, *Pcflo8* was hybridized to *Pc* chromosomes separated by clamped homogeneous electric field (CHEF) and transferred to nitrocellulose in order to map the location of these sequences to the *Pc* genome. From these hybridization studies, we have demonstrated that the *Pcflo8* sequences are present on a single chromosome in the organism and appear to be present on chromosome number 7 (Figure 2).

Pcflo8 steady state mRNA expression is influenced by environmental pH

We have previously reported that a number of *Pc* mRNA transcripts, particularly those involved in cell cycle and cell wall regulation, appear to be influenced by environmental pH, with greatest transcription occurring at lung physiological pH ~7.0 [19–21]. *Pcflo8*

transcription levels also exhibited such variation, but unlike our past studies where greatest expression occurred at pH of 7.0, similar to the lung, we observed a broader range of enhanced PcFlo8 encompassing a range of pH 6–8 (Figure 3). Others have shown that the pH range in fungal biofilms can vary greatly due to metabolic activity within the biofilm flora itself [22].

PcFlo8 protein appears highly expressed in *Pc* trophic life form

Although little is known about *Pc* life cycle regulation due to the inability to culture these organisms *in vitro*, prior observations demonstrate the presence of both smaller trophic forms that lack a rigid cell wall, as well as larger thick-walled cystic forms in the life cycle of the organism. It has been further observed that *Pc* trophic forms exhibit strong attachment to the lung epithelial cells. Additional, *in vitro* modeling investigations further suggest that *Pc* binding to epithelial cells promotes subsequent organism proliferation [1,6,23]. To further evaluate the expression of PcFlo8 over the life cycle of *Pc*, cyst and trophic forms were separated into the respective life forms and lysed [24]. These isolated *Pc* life form proteins were resolved by SDS-PAGE gel and Western blotted performed using a PcFlo8 antibody prepared to a synthetic peptide of the predicted protein (Figure 4, A.). In additional experiments this antibody was used to immunoprecipitate the putative PcFlo8 transcription factor from these enriched and isolated life forms and precipitated proteins were visualized by staining with Coomassie Blue (Figure 4, B.). Both assays demonstrated that the predicted PcFlo8 transcription factor appears abundant in *Pc* trophic forms compared to the cyst forms in the *Pc* life cycle. It also appears from the Western analysis that the trophic form may have more than one isoform of the PcFlo8 protein present as another specific band with slightly smaller MW was noted (Figure 4, A.). These results further suggest that *Pc* trophic forms may utilize a Flo8 regulated adhesin pathway similar to other fungi during attachment to the host.

***Pc* trophic forms contain abundant PcFlo8 protein as determined by immune fluorescence microscopy**

Next, freshly isolated *Pc* life forms were examined for the intracellular presence of the PcFlo8 transcription factor. As we have shown both by Western blotting and immunoprecipitation assays, examination of PcFlo8 in *Pc* life forms by fluorescent microscopy indicated presence of PcFlo8 in the *Pc* trophic forms (Figure 5). Furthermore, when counterstained with the nuclear and chromosome binding reagent DAPI, the PcFlo8 transcription factor appeared to be co-localized to the nucleus, similar to previous observations in *C. albicans* [25]. Overall, these findings suggest that PcFlo8 transcription factor is largely present in *Pc* trophic form. Based on the role of this transcription factor as a mediator of downstream adhesion, invasive growth, and flocculation in other fungi and yeast, it may be postulated to serve important roles in adhesion activity in *Pc* as well.

PcFlo8 can restore downstream transcriptional activation of yeast *FLO11* mRNA

Analysis by semi-quantitative PCR of the potential downstream activation of the major yeast flocculin *FLO1* by PcFlo8 transcription factor expression in the yeast *flo8* mutant strain indicated that indeed, similar to observations by other investigators studying *S. cerevisiae*

Flo8 [26], the Pc transcription factor *Pcflo8* homolog can restore yeast *FLO11* transcription levels similar to the wild-type strain (Figure 6). These observations support that PcFlo8 can regulate the downstream Flo11 adhesin in yeast and thereby drive the subsequent binding to polystyrene and epithelial cells in the *Pcflo8* restored knockout strains.

PcFlo8 promotes fungal adherence to polystyrene

The ability of *S. cerevisiae* to adhere to plastic surfaces has been previously demonstrated, and has been used to study biofilm formation by these yeasts [7]. In *S. cerevisiae* the Flo8 transcription factor regulates adherence by activation of the major adhesion Flo11 [27]. To test whether PcFlo8 could also provide such a function in biofilm formation, *flo8* yeast cells were transformed with *Pcflo8* cDNA and inoculated cells onto wells of a polystyrene microtiter plate. Cells were then incubated for the indicated times points and stained to visualize biofilm formation (Figure 7A). Indeed, *flo8* yeast cells overexpressing *Pcflo8* cDNA, adhered extremely well. In fact, these transformants adhered to a greater overall degree to the inert substrate than the wild-type yeast cells alone as measured by absorbance at 562 nm (Figure 7B). These findings suggest that PcFlo8 can serve as a functional homolog of the *S. cerevisiae* Flo8 transcription factor and promote biofilm formation.

PcFlo8 can regulate invasive growth of fungi

Along with modulating adherence to polystyrene, Flo8 presence in fungi has also been implicated during invasive growth. In the pathogenic yeast *C. albicans*, *FLO8* expression has also been shown to be important in hyphal development in embedded conditions that mimic invasion into the host epithelium [28]. Accordingly, we next determined that overexpression of *Pcflo8* cDNA in the *flo8* *S. cerevisiae* yeast cells promoted an invasive phenotype compared to yeast *flo8* cells containing the control vector alone (Figure 7C). These findings further support activity of PcFlo8 in supporting invasive growth of fungi, and further confirm activity of this molecule as typical of Flo8 proteins in other fungi.

PcFlo8 expression increases adhesion of yeast *flo8* cells to human epithelial cells

In fungal infections, adhesion and invasion of host epithelial and endothelial cells is a thought to be important step in the virulence of pathogenic organisms [29,30]. To determine whether *Pcflo8* expressing *flo8* yeast cells could mediate adhesion to human epithelial cells, transformants were applied to confluent monolayers of human HEK293 epithelial cells. Previous studies with *C. albicans* have shown the effectiveness of this cell line for investigating fungal adhesion [8]. Equal quantities of yeast cell inoculums for all three constructs were applied to the epithelial cells for 1 h at 37°C, followed by washing the monolayers and removing the cells by trypsin digestion. The recovered yeast cells were plated and the numbers of colony-forming units (CFU) determined (Figure 8). *flo8* *S. cerevisiae* cells carrying the empty vector exhibited significantly less binding to the epithelial cell line compared to the wild-type yeast cell alone ($P=0.0007$), whereas *flo8* yeast expressing the *Pcflo8* gene restored adhesive ability to wild-type levels ($P=0.334$, not significantly different compared to control). Thus, *Pcflo8* appears to promote fungal organism adhesion to epithelial cells when heterologously expressed in culture.

DISCUSSION

Pneumocystis jirovecii continues to cause life-threatening pneumonia in patients with AIDS, organ transplantation, cancer, and immune suppression for connective tissue and inflammatory diseases [31]. Indeed, the number of immune compromised patients is increasing due to broader use and greater variety of immunosuppressive regimens [32]. Recent evidence suggests that binding of *Pc* to host epithelium may be an important event in organism proliferation [23,33]. We and others have shown that *Pc* binding to extracellular matrix components of the lung, as well as lung epithelial cells, promotes expression of cell wall remodeling and mating regulatory pathways, as well as proliferation of the organism [4,22,27]. Recent evidence suggests that *Pc* binding events maybe facilitated by an integrin-like molecule in *Pneumocystis* spp. termed PcInt1 [13]. This adhesin homolog also present in *C. albicans* has been demonstrated to participate in organism attachment and virulence [34]. Although this molecule has been implicated in organism binding in *Pc* and *C. albicans*, there are also several other well-characterized adhesins that facilitate *Candida* spp. attachment to host cells and environmental surfaces. For example, both *S. cerevisiae* and in *Candida* spp., utilize a number of “FLO” genes that have been characterized to encode cell-wall glycoproteins, termed adhesins, which mediate binding to inert surfaces as well as mammalian cells [35,7,36]. Recent evidence suggests that these Flo-related proteins, as well as the promoter-activating transcription factor Flo8, are also important for biofilm formation [7].

Based on the importance of these well-defined pathways in *S. cerevisiae* and *C. albicans*, we sought to determine if members of this novel potential adhesion pathway were also present in the *Pc* genome. In the current report, we describe the presence of a PcFlo8 homolog in *Pc*. PcFlo8 exhibited greatest expression in *Pc* trophic forms, the life cycle form that tightly adheres to lung epithelial cells [33,37,38]. In addition, *Pcflo8* was shown to encode a functional protein when heterologously expressed in yeast, supporting organism adherence to environmental surfaces and human cells and promoting invasive fungal growth. Similar to the *S. cerevisiae* Flo8 regulator, the predicted PcFlo8 itself lacks known DNA binding motifs, the hallmarks of most transcription factors, but instead possesses a LUFs domain, and within this region, a LisH motif. Both of these amino acid motifs are found in *S. cerevisiae* and *C. albicans* Flo8 transcription factors and appear necessary for Flo8 function.

In *C. albicans*, mutation of the LUFs domain in Flo8 results in abolishment of hyphal development. Although *Pc* has not been shown to possess a true hyphal form, upon binding to host epithelium the organism does form filopodial protrusions [39,40,23,41]. Interestingly, the activation of this pathway in *Pc* involves the PcSte20 kinase, in a fashion parallel to pseudohyphal growth activation in *S. cerevisiae* [3]. Unfortunately, we are not currently able to mutate the LUFs domain in PcFlo8 and to directly test the effects of this mutation in the *Pc* organism.

In addition to organism-host cell attachment events, Flo8 transcription factors also participate in biofilm formation. Biofilms are defined as groups of microorganisms that adhere to each other and to a host or environmental surfaces [42]. Biofilm associated microbes attach and embed themselves in a self-generated extracellular polymeric substance,

variously consisting of nucleic acid, proteins, and polysaccharides [43]. Biofilms can be generated on both living and nonliving surfaces and have emerging roles in the pathogenesis of many infections. For instance, *Staphylococcus aureus* use biofilm formation to gain entry and persistence in the host via indwelling plastic catheters. In the case of *Legionella* spp. biofilm formation facilitates persistence of the organisms in moist environments [44,45]. Fungi also utilize biofilms as environmental anchors for persistent colonization at the sites of infection, as well as to shield themselves from antifungal therapies [46,47].

Recently, Flo8 has been implicated in *C. albicans* biofilm formation [48]. Additional evidence suggests that *Pc* may form biofilm formation in the lung as well as under culture conditions [6]. Indeed, model *Pc in vitro* biofilms may mimic those observed in the mammalian lung, rendering antifungal therapy less effective [5]. Our current observations may provide further evidence at the genetic level that genomic machinery may exist for *Pc* biofilm formation as it does in *S. cerevisiae* and *C. albicans*. Previous work has suggested that Flo8 homologs in *S. cerevisiae* and *C. albicans* regulate downstream genes important in biofilm formation [7,47]. Our lab attempted likewise to determine if PcFlo8 transcription levels might increase over time in short term culture methods previously reported for *Pneumocystis* spp. [6,5] After culture of *Pneumocystis* spp. in this reported media for 24, 48, and 72 hours, we observed no increase in PcFlo8 protein (data not shown). We have previously observed, however, that although viable organisms can remain in this media for up 72 hours or longer, nucleic acid and protein levels decrease rapidly during the first 6–12 hours. Therefore, until methods become more amenable for longer-term culture and viability of *Pneumocystis*, it will remain undetermined whether PcFlo8 increases overtime in biofilm conditions.

We have previously shown that a *Pc* adhesin, named PcInt1 is expressed highly on the *Pc* tropic form and that when overexpressed in yeast, can lead to organism binding to host extracellular matrix protein such as fibronectin [13]. Preliminary attempts to show interactions of PcFlo8 with PcInt1 have not been revealing. This is not surprising since *C. albicans* Flo8 does not have known interactions with the expression of CaInt1 [8].

Any potential adhesin that PcFlo8 may regulate in *Pneumocystis* currently remains undefined. Interrogations of the available *P. carinii*, *P. murina*, and *P. jirovecii* genome databases (web links noted above) have failed to reveal a conserved homolog to Flo11 in *Pneumocystis* spp. Furthermore, definition of this potential adhesin pathway in *Pc*, will also likely require characterization of additional activation partners of PcFlo8 that are required for transcription factor promoting activity. For instance, phosphorylation of *S. cerevisiae* Flo8 by the protein kinase A catalytic subunit Tpk2 promotes Flo8 binding and activation of the Flo11 promoter [15]. Whether parallel activation systems are also required in *Pneumocystis* spp. is not yet known.

In summary, our current study documents the expression of PcFlo8 in *Pc*, with greatest expression by *Pc* trophic forms. This molecule promotes organism adhesion to inert surfaces and mammalian host cells, as well as invasive growth following heterologous expression in tractable fungi. While direct proof of the role of PcFlo8 in *Pc* adhesion or biofilm formation

is not yet available, this report does provide additional important insights into another potential mechanism of interaction of *Pneumocystis* with the host and the environment.

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The abbreviations used are

Pc	<i>Pneumocystis carinii</i>
RACE	rapid amplification of cDNA ends
ANOVA	analysis of variance
SC	synthetic complete media
CHEF	Contour-clamped homogeneous electric field

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Figure 1.

A. Alignment of the predicted PcFlo8 amino acid sequence with the *S. cerevisiae* Flo8 protein sequence. Sequence alignments of the fungal Flo8 transcription factors was performed with ClustalW (MacVector 8.1.2) demonstrating significant amino acid homology. The sequence data for PcFlo8 are available from GenBank, accession no. KJ790261. **B.** Amino acids alignments of the LUFS domain comparing PcFlo8 and ScFlo8. Identical residues are shaded in dark grey and conserved residues are shaded in light grey.

Figure 2.
Chromosomal location of *Pcflo8*. Pc chromosomes were separated by CHEF. *Pcflo8* hybridizes to a single chromosome.

Figure 3.

Steady state *Pcflo8* mRNA levels are influenced by environmental pH. *Pc* organisms were incubated in Ham's F-12 medium with 10% fetal calf serum at the pH ranges listed for 2 h at 37°C. Northern blot hybridization was utilized next with a labeled 682-bp amplicon as described. The top panel demonstrates hybridization of the the *Pcflo8* probe to the nitrocellulose membrane. The bottom panel displays a photograph of the two *Pc* major ribosomal subunits, confirming equal RNA loading.

Figure 4.

Trophic *Pc* life forms appear to have abundant PcFlo8 protein. **Panel A.** Western blot using the anti-PcFlo8 peptide antibody against separated populations of *Pc* cyst and trophic life form proteins. **Panel B.** PcFlo8 abundance in the *Pc* trophic life form was further determined by immunoprecipitating total *Pc* life form proteins with the PcFlo8 antibody followed by PAGE and Coomassie staining. An equal quantity of healthy uninfected rat lung protein used in both assays failed to display any immune reactivity with the anti-PcFlo8, confirming the specificity of the antibody employed.

Figure 5.

PcFlo8 transcription factor protein is present and colocalizes to the nucleus in intact *Pc* trophic forms. (**A, D**) Phase microscopy images of trophic forms (100X magnification). (**B, E**) DAPI staining of *Pc* trophic forms. (**C**) Fluorescence microscopy staining with antibody generated against the predicted PcFlo8 demonstrates specific staining within the nucleus of trophic forms. (**F**) Fluorescence microscopy staining with isotype IgG control antibody.

Figure 6.

Pcflo8 causes induction of yeast *FLO11* gene expression in respective *S. cerevisiae* strains. RNAs of the listed strains were isolated and compared after RT-PCR followed by semiquantitative PCR using *Taq* polymerase. The PCR reactions were compared by 3% TAE-agarose gel electrophoresis. As a loading control *ACT1* transcripts levels were also analyzed.

Figure 7.

PcFlo8 promotes adherence to polystyrene and fungal invasion of *S. cerevisiae* into agar.

(A.) Adherence to plastic was conducted as noted. After overnight growth, *S. cerevisiae* cell numbers were equalized by OD₆₀₀ nm and confirmed by plating. Cells were transferred to microtiter plates and incubated for the indicated times. (B.) Final absorbance of the microtiter plates was conducted after staining and these numbers plotted against time. *PcFlo8* induced significant adhesion of *S. cerevisiae* cells to plastic as compared to the *flo8* (deficient) yeast cells containing control vector alone (*P < 0.05 comparing to *flo8* deficient yeast containing empty vector to *flo8* yeast containing *PcFlo8* at all times 15 minutes and greater). (C.) Strains with the indicated genotypes were further tested for invasive growth

into agar. Plates were photographed before and after extensive washing with water. *Pcflo8* expression in *flo8* *S. cerevisiae* supported significant invasion of agar plates.

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Figure 8.

PcFlo8 promotes adhesion of *S. cerevisiae* cells to 293 human epithelial cells. The respective transformants were grown overnight at 30°C in 2% galactose and then applied in equal numbers to the 293 cell monolayer. After extensive washing with 1X PBS, both the monolayer and yeast cells were detached by trypsin and applied to SC plates without uracil and containing 2% galactose. *PcFlo8* expression in the *flo8* yeast cells induced significant adherence phenotypes similar to the wild-type levels. Overall the one way ANOVA across the three conditions was <0.0001. (*Denotes $P < 0.0007$ comparing the *Flo8* deficient *flo8* yeast containing empty vector to wildtype control. **Denotes $P = 0.334$ comparing the *Flo8* deficient *flo8* yeast transformed with *PcFlo8* vector to wildtype control, not significantly different).